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Detection of Cell Wall Mannoprotein Mp1p in Culture Supernatants of *Penicillium marneffei* and in Sera of Penicilliosis Patients

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Mannoproteins are important and abundant structural components of fungal cell walls. The *MP1* gene encodes a cell wall mannoprotein of the pathogenic fungus *Penicillium marneffei*. In the present study, we show that Mp1p is secreted into the cell culture supernatant at a level that can be detected by Western blotting. A sensitive enzyme-linked immunosorbent assay (ELISA) developed with antibodies against Mp1p was capable of detecting this protein from the cell culture supernatant of *P. marneffei* at 10^4 cells/ml. The anti-Mp1p antibody is specific since it fails to react with any protein-form lysates of *Candida albicans*, *Histoplasma capsulatum*, or *Cryptococcus neoformans* by Western blotting. In addition, this Mp1p antigen-based ELISA is also specific for *P. marneffei* since the cell culture supernatants of the other three fungi gave negative results. Finally, a clinical evaluation of sera from penicilliosis patients indicates that 17 of 26 (65%) patients are Mp1p antigen test positive. Furthermore, a Mp1p antibody test was performed with these serum specimens. The combined antibody and antigen tests for *P. marneffei* carry a sensitive of 88% (23 of 26), with a positive predictive value of 100% and a negative predictive value of 96%. The specificities of the tests are high since none of the 85 control sera was positive by either test.

Disseminated systemic fungal infections frequently occur in immunocompromised patients (22). Since many of them fail to produce sufficient levels of specific antibodies that can be detected by serological tests (22), the diagnosis of systemic fungal infections often depends on the detection of fungal products or fungal antigens.

Analysis of fungal cell wall components revealed the presence of glucan, chitin, and mannoproteins. Cell wall mannan and mannoproteins are abundant and important fungal antigens that represent up to 25% of the total cell wall mass (9, 10, 15). Mannoproteins can be solubilized and removed from the cell surface by denaturation (19) or with reducing agents (13). In addition, the detection of mannan or galactomannan was shown to be useful in the diagnosis of systemic fungal infections such as systemic candidiasis (5, 8) and systemic aspergillosis (14, 21) by the detection of antigenemia.

In the study described here, we explored the possibility of detecting specific mannoproteins in the circulating blood as an alternative approach to the molecular diagnosis of systemic fungal infections. Previous protein sequence analysis of fungal mannoproteins revealed the presence of a secretory signal peptide in them (20). Because they are located on the fungal cell wall and can readily be removed from the cells, we rationalized that they may also be secreted from the cells as the fungi grow and divide. The detection of a specific mannoprotein for the diagnosis of a systemic fungal infection by the detection of antigenemia may have certain advantages. The preparation of the antigen can be more reproducible since it depends on the purification of a specific recombinant protein. In addition, an enzyme-linked immunosorbent assay (ELISA) can be developed with antibodies to a specific mannoprotein. Such an ELISA is better defined and may therefore be more quantitative, sensitive, and specific.

Penicillium marneffei is an important dimorphic pathogenic fungus that is endemic in Southeast Asia and southern parts of China. It causes a disseminated and progressive disease, penicilliosis marneffei. The disease occurs primarily in AIDS patients, although it has also been reported to occur in immunocompetent patients (6, 7, 16, 17, 24). A gene, *MP1*, that encodes an antigenic cell wall mannoprotein, Mp1p, has been cloned (1). Further analysis indicated that more than 80% of the penicilliosis patients in Hong Kong who were seropositive for human immunodeficiency virus (HIV) had significant levels of antibody against Mp1p (2). However, a much reduced proportion (about 40%; 6 of 14) of HIV-positive with penicilliosis patients in Thailand tested positive by the same test (unpublished data), perhaps due to the fact that some patients might not produce significant levels of specific antibody. An earlier study done by Kaufman et al. (11) with concentrated filtrate antigens showed that only 2 of 17 penicilliosis patients had detectable levels of specific antibody. However, approximately 60 to 70% of these patients were positive by the tests for antigenemia (immunodiffusion or latex agglutination test) developed with antibodies raised against total cell lysate filtrates of *P. marneffei* cells (11), indicating the presence of fungal antigens in the sera of patients who were antibody test negative.

The present study reports on an ELISA-based test for antigenemia that detects the Mp1p mannoprotein of *P. marneffei* for the serological diagnosis of penicilliosis. First, mannoprotein Mp1p was detected in the cell culture supernatant of *P. marneffei* by Western blotting. The anti-Mp1p antibody is specific for *P. marneffei* since no cross-reaction was observed between cell lysates of *Candida albicans*, *Histoplasma capsulatum*, or *Cryptococcus neoformans* and the specific antibodies used to set up the Mp1p ELISA. Next, a sensitive and quan-

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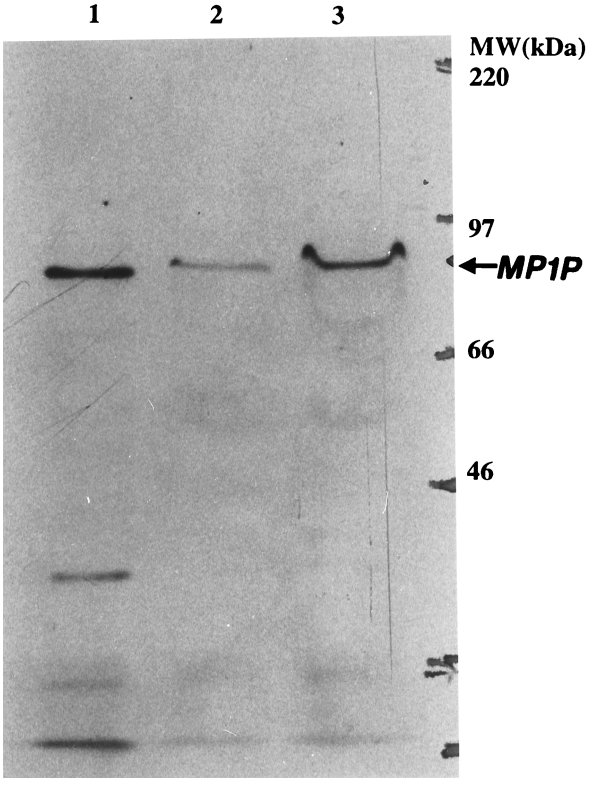

FIG. 1. Western blot analysis of Mp1p in the culture supernatant of *P. marneffei*. Lane 1, 20 μg of a cell lysate of *P. marneffei*; lane 2, 5 μl of a concentrated culture supernatant; lane 3, 10 μl of a concentrated culture supernatant.

titative ELISA-based antigen test was developed to detect the presence of Mp1p and to quantitate the amount of Mp1p in the cell culture supernatants of *P. marneffei*. Furthermore, the antigen detection test was found to be specific for *P. marneffei* since the cell culture supernatants of *C. albicans*, *H. capsulatum*, and *C. neoformans* were all negative by the ELISA. Lastly, this Mp1p antigen test complements an ELISA-based Mp1p antibody test for the diagnosis of systemic penicilliosis.

MATERIALS AND METHODS

Strains and growth conditions. *P. marneffei* PM4 and *C. albicans* NGY10 were used previously (1, 2). *H. capsulatum* ATCC 26032 was obtained from the American Type Culture Collection (Rockville, Md.), and *C. neoformans* is a clinical isolate (from patient 96M0112693) from Queen Elizabeth Hospital, Hong Kong. Fungal cells were first grown on YPD plates (1% yeast extract, 2% Bacto Peptone, 2% glucose, 1% agar) at 30°C. Fungal cultures were obtained by inoculating fungal cells from plates into RPMI medium (Gibco-BRL, Gaithersburg, Md.) and were further shaken at 37°C for 1 to 3 days.

Human and animal sera. Sera were obtained from patients with penicilliosis that was documented by examination of bone marrow, spleen, skin, or lymph node biopsy specimens and/or blood culture results. Serum specimens were obtained from penicilliosis patients ($n = 2$ patients from Queen Mary Hospital, Hong Kong) without HIV infection or other conditions of immunodeficiency. Additional serum specimens were obtained from HIV-positive patients with penicilliosis from Hong Kong ($n = 10$ patients from Queen Elizabeth Hospital) and Thailand ($n = 14$ patients from Chiang Mai University, Chiang Mai, Thailand). The negative control sera obtained from subjects at Queen Mary Hospital were from healthy blood donors ($n = 40$) and patients with documented tuberculosis ($n = 29$), and the negative control sera from Chiang Mai University ($n = 16$) included 6 serum samples from HIV-positive AIDS patients without penicilliosis. Guinea pig and rabbit anti-Mp1p antibodies were produced as described previously (1).

Preparation of cell lysate and cell culture supernatant. Fungal cells were collected by centrifugation (1) and were resuspended in lysis buffer (25 mM Tris-HCl, [pH 7.5], 100 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After disruption of the cells by sonication, the lysed cells were centrifuged at 13,000 rpm in a microcentrifuge for 15 min. The supernatants were collected as cell lysates.

To obtain culture supernatants of *P. marneffei*, the cells were grown in 500 ml of RPMI to an optical density at 600 nm (OD_{600}) of 1. They were precipitated and resuspended in 20 ml of RPMI and were shaken at 37°C for an additional 2 h. After centrifugation, the culture supernatant was passed through a 0.45-μm-pore-size filter (Corning Inc., Corning, N.Y.). The proteins in the supernatant were precipitated by adding 80 ml of saturated $(NH_4)_2SO_4$, and the protein pellet was resuspended in 500 μl of H_2O.

Western blot analysis. Approximately 20 μg of proteins from the cell lysates or 5 to 10 μl of concentrated culture supernatant of *P. marneffei* was loaded onto each lane of a sodium dodecyl sulfate–10% polyacrylamide gel and subsequently the proteins were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, Calif.). The blot was incubated with a 1:1,000 dilution of guinea pig or rabbit anti-Mp1p antibodies, and the proteins were then detected with an enhanced chemiluminescence fluorescence system (Amersham Life Science, Buckinghamshire, England) (1).

Serological test. To produce ELISA plates for the antigen test for penicilliosis, Nunc (Roskilde, Denmark) immunoplates were coated with a guinea pig anti-Mp1p antiserum at a 1:5,000 dilution for 12 h and were further blocked in phosphate-buffered saline with 2% bovine serum albumin. The serological test was performed as described previously (4). Specifically, fixed amounts of purified Mp1p proteins, diluted fungal culture supernatants, or 1:20-diluted human serum specimens were added to the wells and the plates were incubated at 37°C for 2 h. After the wells were washed, the rabbit anti-Mp1p antiserum was added at a 1:500 dilution and the plates were incubated at 37°C for 1 h. After the wells were washed, 1:2,000-diluted alkaline phosphatase-conjugated goat anti-rabbit antibody was added. Detection was carried out with *p*-nitrophenyl phosphate substrate (Sigma Immuno Chemicals, St. Louis, Mo.).

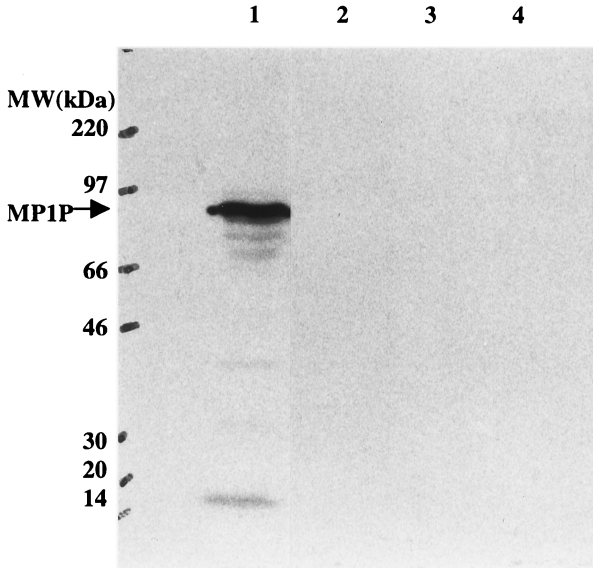

FIG. 2. Mp1p is specific for *P. marneffei* cells. Approximately 20 μg of each of the cell lysates from *P. marneffei* (lane 1), *C. albicans* (lane 2), *C. neoformans* (lane 3), and *H. capsulatum* (lane 4) was loaded into each lane for Western blot analysis with a specific rabbit anti-Mp1p antibody.

RESULTS

Detection of Mp1p protein in culture supernatants of *P. marneffei* cells by Western blotting. To examine cell culture supernatants of *P. marneffei* for the presence of the Mp1p mannoprotein, the supernatant was concentrated and 5 μl (Fig. 1, lane 2) or 10 μl (Fig. 1, lane 3) of the concentrated samples was loaded onto a sodium dodecyl sulfate-protein gel for Western blot analysis with specific antiserum against Mp1p. As a positive control for Mp1p, 20 μg of *P. marneffei* cell lysate was also loaded onto the same gel (Fig. 1, lane 1). The Western blot was probed with a guinea pig anti-Mp1p antibody. The results of the Western blot analysis presented in Fig. 1 revealed the presence of the Mp1p protein with a molecular mass of

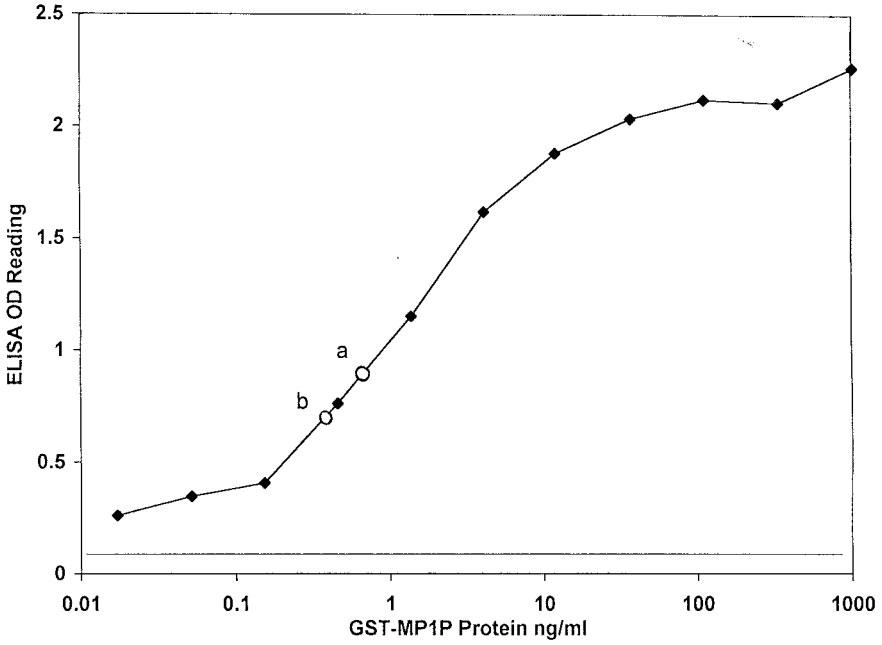

FIG. 3. Standard curve for Mp1p and determination of Mp1p concentration in culture supernatants of *P. marneffei*. The standard curve of the antigen ELISA was determined with a purified recombinant Mp1p protein. Two culture supernatants of *P. marneffei* with final densities of 2×10^6 (a) and 8.6×10^5 (b) cells/ml were diluted 1 to 9 and were subjected to the antigen ELISA. The OD_{405} values obtained by ELISA were plotted to determine the Mp1p protein concentrations in the two supernatants (3.8 and 3.4 ng/ml, respectively).

about 90 kDa in both the cell lysate (Fig. 1, lane 1) and the concentrated cell culture supernatant (Fig. 1, lanes 2 and 3). The size of the protein is significantly greater than the predicted molecular mass of 46 kDa on the basis of its amino acid sequence, and this is likely to be due to the mannoglycosylation of Mp1p (1).

Antibody against Mp1p failed to recognize any reactive protein in other pathogenic fungi. Previous protein sequence analysis of Mp1p revealed that Mp1p is a unique protein with no homologue in the entire GenBank database. No homologue can be identified when Mp1p was used in a BLAST search against the complete genome of the yeast *Saccharomyces cerevisiae* (1). To exclude the cross-reactivity between anti-Mp1p antibody and proteins of other pathogenic fungi, cell lysates were made from *C. albicans*, *C. neoformans*, and *H. capsulatum*, which are frequent causes of infections in AIDS patients. The cell lysates were analyzed by Western blotting with a rabbit anti-Mp1p antibody. The results in Fig. 2 indicated that only the lysate of *P. marneffei* contains a reactive band of 90 kDa (lane 1), whereas none of the other fungal pathogens has cross-reacting protein (lanes 2 to 4). Thus, no cross-reacting protein from the cell lysates of three medically important pathogenic fungi can be detected with the anti-Mp1p antibody.

Development of an ELISA-based antigen test for detection of Mp1p. The presence of Mp1p in cell culture supernatants raised the possibility that the protein antigen could be detected in serum specimens from infected patients. To develop a sensitive test for the detection of mannoprotein Mp1p for the diagnosis of penicilliosis, two types of polyclonal antibodies were obtained from both rabbits and guinea pigs after immunization with purified glutathione *S*-transferase (GST)–Mp1p fusion protein. A sandwich ELISA system was then generated with a guinea pig anti-Mp1p antiserum as the capturing antibody and a rabbit anti-Mp1p antiserum as the detection antibody. Since most of the immunoreactivity of the capturing anti-Mp1p antibody from the guinea pig is specific for the Mp1p portion of the fusion protein, the test should primarily detect Mp1p.

By using a serial dilution of the purified recombinant Mp1p protein, a standard curve for the Mp1p antigen test was obtained, as shown in Fig. 3. Bovine serum albumin was used to establish the baseline for the test at an OD_{405} of 0.0725. The cutoff value was set to be 0.145, which is equal to twice the OD_{405} for bovine serum albumin. The largest dilution of the purified recombinant Mp1p protein gave a concentration of 17 pg/ml. By the ELISA the OD_{405} at this dilution is 0.25, which is well above the cutoff value of 0.145. Therefore, the lower limit of the detection sensitivity of the test is 17 pg/ml for the GST-Mp1p protein. The standard curve is linear for Mp1p at concentrations of between 0.1 to 10 ng/ml, therefore allowing the quantitation of the Mp1p protein.

To detect the presence of Mp1p and to determine the concentration of it in cell culture supernatants of *P. marneffei*, the cells were grown to densities of approximately 8.6×10^5 and 2×10^6 cells/ml in RPMI with 10% serum. Both culture supernatants were collected after centrifugation and were passed through 0.45-μm-pore-size filters to remove all cells. Threefold serial dilutions were made, and the diluted culture supernatants were subjected to the Mp1p antigen ELISA. At a 1:9 dilution, these two culture supernatants gave OD_{405} values of 0.738 and 0.871, respectively, by the ELISA. After comparison with the standard curve obtained with purified recombinant Mp1p protein, the Mp1p protein concentrations of the two culture supernatants described above were determined to be 3.4 and 3.8 ng/ml at cell densities of 8.6×10^5 and 2×10^6 cells/ml, respectively. The concentrations of the Mp1p proteins present in both culture supernatants are approximately 200 times greater than the lower limit of sensitivity of the Mp1p antigen test (17 pg/ml). This estimation is consistent with the results from another limited-dilution experiment of a culture supernatant of *P. marneffei* (Fig. 4). In that experiment, a pos-

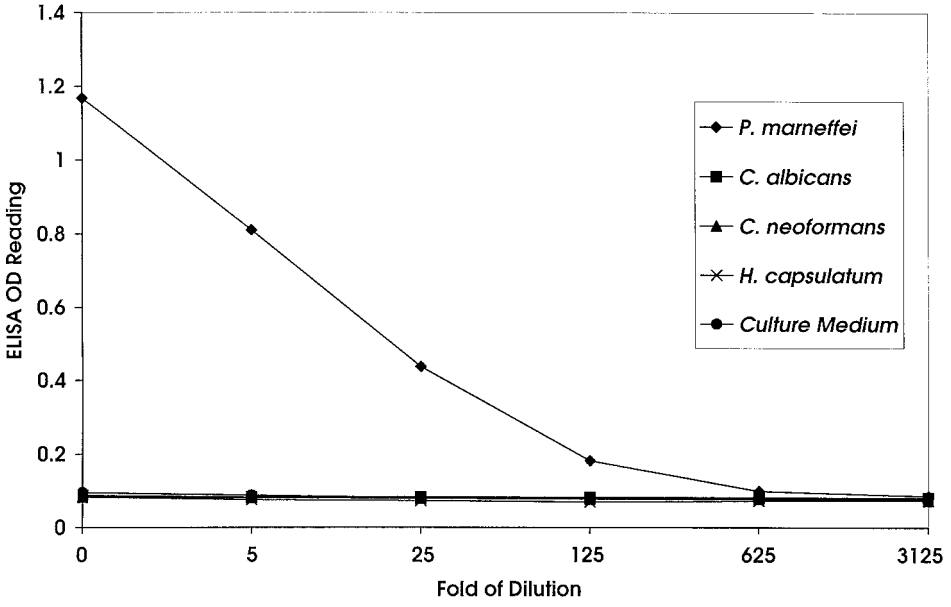

FIG. 4. Mp1p antigen ELISA is specific for *P. marneffei*. Cell culture supernatants were obtained from cultures of *P. marneffei*, *C. albicans*, *C. neoformans*, and *H. capsulatum* grown to densities of about 1×10^6 to 2×10^6 cells/ml and were subjected to the Mp1p antigen ELISA. The positive ELISA values are restricted to the supernatants of *P. marneffei* cells. Notice that the largest dilution for the *P. marneffei* culture supernatant with a positive OD_{405} value is 1:125.

itive ELISA signal can be detected with a 1:125 dilution but not with a 1:625 dilution. Thus, a positive ELISA signal could be expected from the cell culture supernatant of *P. marneffei* with 10^4 cells/ml. It was also estimated that the sensitivity of this ELISA-based antigen test is about 1,000 times greater than that of the previous Western blot assay.

Fungal specificity of the antigen test for penicilliosis. To determine the specificity of the Mp1p ELISA, culture supernatants were obtained from important pathogenic fungi, *P. marneffei*, *C. albicans*, *H. capsulatum*, and *C. neoformans*, grown to densities of between 1×10^6 and 2×10^6 cells/ml. Serial dilutions of the supernatants were produced and were analyzed by this test. The result of the ELISA is presented in Fig. 4. Only the *P. marneffei* culture supernatant gave a positive signal. None of the other culture supernatants has OD_{405} values greater than 0.1. In fact, none of those values is different from that for the culture medium alone. The results indicate that this ELISA-based antigen test is specific for *P. marneffei* and has no cross-reactivity with other important pathogenic fungi.

Detection of Mp1p protein in serum specimens from penicilliosis patients. A clinical evaluation of the Mp1p ELISA was carried out. To establish the baseline of this assay, serum specimens from 40 healthy blood donors were tested (Fig. 5). The mean OD_{405} value for these specimens as determined by the ELISA was 0.066, with a standard deviation of 0.0055. The cutoff OD_{405} value of the ELISA was then defined as follows: cutoff = mean + 10 × standard deviation = 0.121. It was set sufficiently high to eliminate the occurrence of false-positive results by the test.

Serum specimens were obtained from a total of 26 penicilliosis patients confirmed by either blood culture or examination of biopsy specimens. Among 12 patients from Hong Kong, 2 who were free of HIV had significant levels of antibody to against Mp1p, and among 10 others who were HIV seropositive, 8 were positive for antibody to Mp1p (2). Another 14 serum specimens were obtained from Thai patients who were HIV seropositive. The results of the Mp1p antigen test presented in Fig. 5 indicate that among the HIV-seropositive patients, 6 of 10 patients (60%) from Hong Kong and 11 of 14 Thai patients (79%) were positive by the test. Interestingly, neither patient who was HIV seronegative had a positive result by this antigen test. The antigen test has an overall sensitivity of 65% (17 of 26).

In addition to the antigen test, Mp1p antibody tests were also performed with all serum specimens listed in Fig. 5. The antibody test was done as described previously (2). A detailed analysis of both Mp1p antibody and Mp1p antigen test results is presented in Table 1. The results indicated that only 38% (10 of 26) were positive by both the antibody and the antigen tests. However, half of the patients were either antibody positive (6 of 26) or antigen positive (7 of 26). Therefore, the proportion of patients who are positive by at least one of the tests is 88% (23 of 26).

The specificity of the Mp1p antigen test is expected to be higher than that of a Western blot assay since the antigens need to be recognized by two different antibodies to produce a positive signal. In this study, the Mp1p antigen test results with serum specimens from 40 healthy blood donors, 16 Thai people (6 of whom were HIV seropositive and who had infections other than penicilliosis), and 29 patients with tuberculosis indicated that none of the subjects in the three negative control groups produced a positive signal, indicating the high specificity of the test. Similarly, all 85 control serum specimens were negative by the Mp1p antibody test (data not shown). Therefore, the specificities of both tests are 100%.

DISCUSSION

Mp1p is an abundant cell wall mannoprotein with a secretion signal peptide, and it is unique to *P. marneffei*. Western blot analysis of cell culture supernatants of *P. marneffei* with an anti-Mp1p antibody reveals that Mp1p can readily be detected in these supernatants. A sensitive and specific sandwich Mp1p ELISA-based antigen test was developed with two different polyclonal antibodies against Mp1p for the detection of Mp1p protein in serum specimens. The test can detect and quantitate

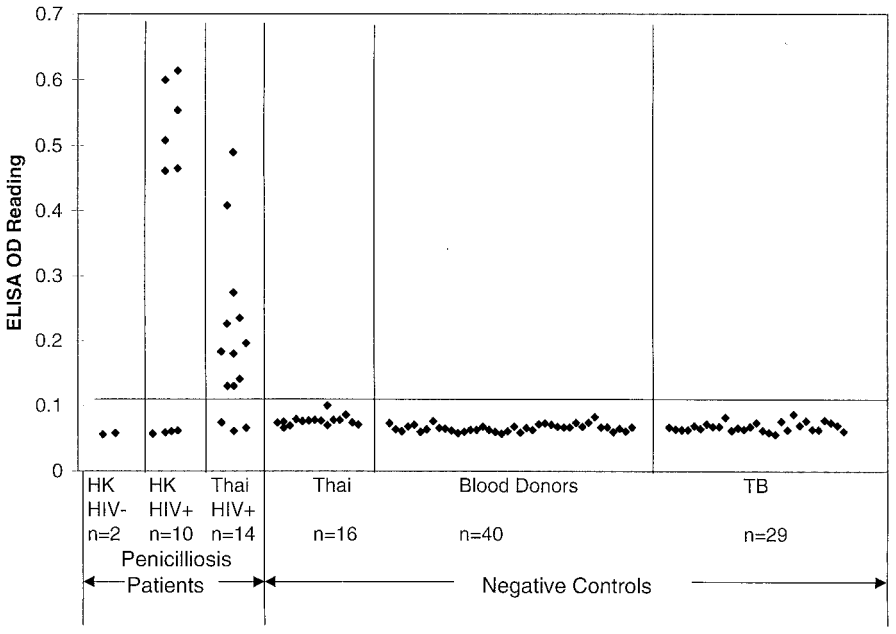

FIG. 5. Evaluation of sensitivity and specificity of the *P. marneffei* antigen test for the detection of penicilliosis in patients.

Mp1p in cell culture supernatants of *P. marneffei*. A clinical evaluation of serum specimens from penicilliosis patients indicates that 65% (17 of 26) of the patients have detectable levels of Mp1p antigen in their circulating blood. The Mp1p antigen assay is specific since no false-positive result was obtained for 85 specimens from healthy blood donors, AIDS patients without penicilliosis, and patients with tuberculosis. This test appears to complement an antibody test previously developed for the detection of anti-Mp1p antibodies (2). Thus, the detection of the specific cell wall mannoprotein Mp1p can be of value in the serological diagnosis of penicilliosis.

Because the majority of penicilliosis patients are severely immunocompromised because they have AIDS, the Mp1p antigen detection test is particularly important for the diagnosis of the infection in these patients. The analysis of anti-Mp1p antibodies with Thai patients revealed that only 42% of the patients (6 of 14) have detectable levels of anti-Mp1p antibody. Although another 50% of the Thai patients (7 of 14) were antibody test negative, they were positive by the antigen test.

TABLE 1. Performance of antigen and antibody ELISA for diagnosis of *P. marneffei* infection in patients with documented penicilliosis

Serological test result[a]	No. of patients	
	Penicilliosis patients (n = 26)	Negative controls (n = 85)
Ag+, Ab+	10	0
Ag+, Ab−	7	0
Ag−, Ab+	6	0
Ag−, Ab−	3	85
Positive by either test[b]	23	0

[a] Ag+, antigen test positive; Ab+, antibody test positive; Ag−, antigen test negative; Ab−, antibody test negative.

[b] The sensitivity, as indicated by a positive result by either test, is 88% (23 of 26), with a specificity of 100% (0 of 85). The test results for the combination of both antigen and antibody tests have a positive predictive value of 100% and a negative predictive value of 96%.

This antibody test result for Thai penicilliosis patients with AIDS is different from our previous observation (24) that all penicilliosis patients without AIDS had significant levels of antibodies to *P. marneffei*. It may be suggested that perhaps many of the AIDS patients tested in the present study did not produce detectable levels of antibody to Mp1p.

The antigen test, however, is not completely sufficient. Of the nine patients who were antigen test negative, six, including both immunocompetent penicilliosis patients, were antibody test positive, suggesting that the Mp1p antigen may be removed more effectively in hosts with intact immune systems. On the basis of this result, we suggest that both tests be performed for patients in whom penicilliosis is suspected. The antibody test may be more sensitive for patients who are immunocompetent or who have better humoral immune systems, while the antigen test would be more useful for patients who have more compromised immune systems. The combined tests for antibody and antigen have a sensitivity of 88%, with a positive predictive value of 100% and a negative predictive value of 96%.

The mannoprotein Mp1p-based antigen test described here has several unique features. The absolute sensitivity of the ELISA is high, about 20 pg/ml. This is 50 times greater than the sensitivity reported in another study for a sandwich ELISA for the detection of circulating galactomannan in patients with invasive aspergillosis (14). The difference in sensitivity may be due to the fact that Mp1p is a highly immunogenic protein antigen, whereas galactomannan was used in the previous study. In addition, the test shows very good specificity both in vitro with fungal cultures and in vivo with human serum specimens. The high specificity is likely due to the fact that a purified recombinant protein antigen was used for antibody production. In contrast, antigen tests developed against crude fungal antigens may have significant cross-reactivities with several pathogenic fungi (23). Furthermore, the Mp1p antigen ELISA described here is also quantitative. Such quantitation of a circulating antigen may be important as a prognostic indicator because it may reflect both the fungal load and the host's

ability to clear the fungal antigen. Also, such quantitation may be of a value in the monitoring of antifungal therapy for penicilliosis patients.

The study presented here may have implications on the future development of means of molecular diagnosis of systemic fungal diseases in immunocompromised patients. This is the first evaluation of a test that detects a specific mannoprotein in immunocompromised patients. As a cell wall protein, Mp1p has a signal peptide that permits its translocation across the cytoplasmic membrane to the cell wall. Perhaps as the result of being a cell wall protein, Mp1p can be detected at a high concentration in culture supernatants of *P. marneffei*. One might expect that a similar result could also be true for other cell wall mannoproteins. Previous studies with cell wall proteins indicated several unique features of fungal cell wall mannoproteins that include a signal peptide, a serine- and threonine-rich region for O glycolation, and a glycophosphatidylinositol (GPI) membrane attachment motif (12, 18). A number of genes for mannoproteins were identified from the yeast *S. cerevisiae* in gene cloning and function studies. The completion of the sequencing of the *S. cerevisiae* genome allows the further identification of a large number of cell wall mannoprotein genes (3). The genome sequencing projects with *Candida* and *Aspergillus* will undoubtedly reveal their cell wall mannoprotein genes. It should be pointed out that although there is a conservation of motifs among the cell wall mannoproteins, many of them are very different from each other at the protein sequence level. In fact, most of them have no protein sequence homology with each other, and therefore, diagnostic tests that detect mannoproteins can be very specific, as in the case of Mp1p. Our work validates the approach of developing tests for the detection of antigenemia with a cell wall mannoprotein and streamlines the process of development of such a test system.

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